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Sub-mitochondrial localization of the catalytic subunit of pyruvate dehydrogenase phosphatase

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Abstract Using a specific antibody against the PDP catalytic subunit, PDPc, precise localization of this subunit in mitochondria was performed. Sub-fractionation of purified mitochondria by controlled swelling processes led to the isolation of outer membranes, matrix space and inner membrane vesicles which were purified on a sucrose density gradient. In this study, we demonstrated that PDPc was not recovered as a soluble protein in the matrix space but was associated with the inner membrane. Moreover, Triton X-114 phase partitioning performed on inner membranes showed that PDPc behaved both as a hydrophilic and as a hydrophobic protein, thus suggesting two different forms of this enzyme.

Key words: PDH complex; PDH phosphatase; Mitochondrial membrane; Triton X-114

1. Introduction

The pyruvate dehydrogenase multienzymatic complex (PDH), which catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA, is an important converter enzyme between the glycolytic pathway and either the tricarboxylic acid cycle with ATP synthesis or lipid biosynthesis. Such an enzymatic complex localized at a crucial metabolic point needs to be acutely controlled. PDH complex activity regulation is achieved by both the reaction products and reversible phosphorylation. The PDH complex undergoes an interconversion between an active non-phosphorylated form (PDHa) and an inactive phosphorylated form (PDHb) [1]. The phosphorylation is catalyzed by a specific kinase tightly bound to dihydrolipoyl transacetylase (E2 component). Dephosphorylation and subsequent activation of the PDH is catalyzed by a Mg^{2+}-dependent and Ca^{2+}-stimulated serine/threonine phosphatase. This phosphatase, which has proven over the years to be somewhat refractory to purification, has finally been purified from different sources [2–5]. This enzyme is a heterodimer [2–4], which consists of a cloned catalytic subunit, PDPc, of M_r 52 600 which shares sequence homology with the protein phosphatase 2C family [6], and a regulatory subunit, PDPr, of M_r 95 800 which is a flavoprotein thought to be involved in the insulin-induced signaling pathway, leading to the activation of the PDH complex [7]. In eukaryotic cells, the PDH complex is localized in mitochondria within the inner membrane-matrix compartment. After mitochondria disruption and separation of mitochondrial membranes by centrifugation, the PDH complex remains partially bound to the inner membrane [8,9]. Up to now, no precise localization of this enzymatic complex in mitochondria and no topological studies of this complex have been described although a better understanding of the organization of this enzymatic complex within mitochondria should facilitate studies on regulation of this enzyme. In this study, using a controlled sub-fractionation of purified liver mitochondria and an antibody raised against the amino-terminal sequence of PDPc, we prove that PDPc does not exist as a soluble matrix protein but tightly interacts with mitochondrial inner membrane, where this enzyme is uniformly distributed.

2. Materials and methods

2.1. Preparation and purification of mitochondrial membranes

Mouse liver mitochondria, outer membrane vesicles and mitoplasts were prepared as previously described [10]. Mitoplasts were subjected to swelling in 1 mM potassium phosphate buffer pH 7.4 at 4°C, and centrifugation at 100 000 × *g* for 1 h led to obtaining inner membrane vesicles (pellet) and matrix space (supernatant) which was lyophilized and stored at −20°C. Inner membrane vesicles were purified on a discontinuous sucrose gradient (41%, 44.5%, 47.9%, 51.3% and 61.5%). After centrifugation at 25 000 rpm (rotor SW28) for 2 h, four membranous fractions were collected, washed in 10 mM Tris-HCl pH 7.5 buffer and stored in liquid nitrogen. Protein concentration in the different fractions was determined according to the Gornall's procedure [11]. To detect ultramicro amounts of proteins, the method of Schaffner and Weissmann [12] was used.

2.2. Marker enzyme assays

Monoamine oxidase (EC 1.4.3.4), as outer membrane marker enzyme, cytochrome *c* oxidase (EC 1.9.3.1), as inner membrane marker enzyme, basic 5′-nucleotidase (EC 3.1.3.5), as plasma membrane marker enzyme and glucose-6 phosphate phosphatase (EC 3.1.3.9), as rough endoplasmic reticulum marker enzyme were assayed as previously described [10]. Citrate synthase (EC 4.1.3.7), as matrix space marker enzyme was assayed according to Srere [13].

2.3. Electrophoretic procedures

For mitochondrial proteins, SDS-PAGE was performed under reducing conditions according to the method of Laemmli [14], using a 10% separating gel at a voltage of 200 V for 45 min. Two-dimensional electrophoresis was carried out according to O'Farrel [15] with 1.6% of ampholines pH 5–7 and 0.4% of ampholines pH 3–10 for 5 h at 750 V using a BioRad apparatus. Pi range was calibrated with a calibration kit (BioRad).

2.4. Immunological procedures

2.4.1. Production of a specific antibody against amino-terminal sequence of PDPc. This specific antibody was prepared by Covalab using the amino-terminal peptide sequence (ASTPQKFYLTPPQVN) of the mature catalytic subunit of bovine PDPc described by Lawson et al. [6] and used for rabbit immunization. Before injection, 5 ml of

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Abbreviations: PDH, pyruvate dehydrogenase; PDP, pyruvate dehydrogenase phosphatase; PDPc, catalytic subunit of PDP; N-PDPc, amino-terminal sequence of PDPc; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TSN buffer, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Nonidet P40; BSA, bovine serum albumin

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blood was collected as preimmune control serum. Immunization was performed by a subcutaneous and muscular multi-injection technique each time with 0.5 mg of synthetic peptide coupled to a carrier protein and emulsified with an equal volume of Freund's adjuvant for the first immunization and without adjuvant for the booster injections. Diluted immune serum was used as a source of antibodies without further purification.

2.4.2. Immunoblotting. SDS-PAGE or 2D-electrophoresis separated proteins were electro- transferred onto nitrocellulose sheets. The sheets were incubated overnight at +4°C in TSN buffer containing 3% BSA to block non-specific antibody binding. The sheets were then incubated for 90 min in the presence of anti-N-PDPc antibody diluted to 1/3000 in TSN containing 0.25% BSA (buffer I), and washed three times in TSN. The immunological study was performed using anti-rabbit IgG peroxidase-labeled (diluted to 1/2000 in buffer I) and chemiluminescent visualization (Covalab kit).

2.5. Protein staining

Proteins were stained with a silver nitrate method using the Silver Stain Plus kit (BioRad) on SDS-PAGE and with Ponceau red on nitrocellulose sheets.

2.6. Treatment with increasing salt concentrations

Samples of IM_3 fraction (25 μg) were incubated for 30 min at +4°C in 50 μl of 10 mM Tris-HCl pH 7.4 containing increasing concentrations of NaCl, from 50 to 500 mM. After centrifugation, solubilized proteins (supernatant) and membranous proteins (pellet) were collected and acetone precipitated.

2.7. Triton X-114 phase partitioning of proteins

Mitochondrial membranes (100 μg proteins in 100 μl) were incubated in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA containing 2% of precondensed Triton X-114 [16] at +4°C for 15 min and phase partitioning was performed according to the method described by Bordier [16], slightly modified. Briefly, after incubation at 37°C for 10 min, the different samples were centrifuged at 10 000×*g* for 1 min in a microfuge. The phases were separated and 50 μl of 4% ice-cold Triton X-114 and 50 μl of ice-cold theoretical upper phase containing 0.06% Triton X-114 were added to the upper and lower phase, respectively. After a second phase partitioning, aqueous and detergent-rich phases were pooled and proteins of each phase were acetone precipitated.

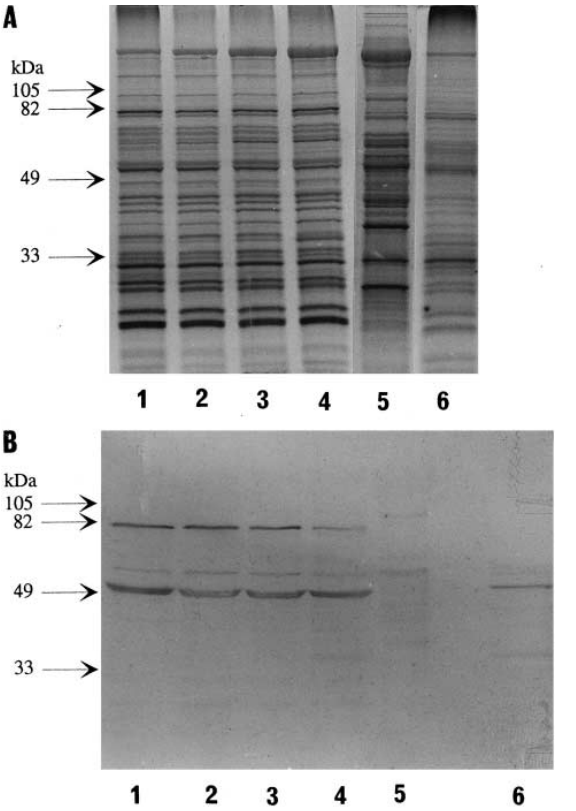

Fig. 1. SDS-PAGE and Western blot analysis of sub-mitochondrial fractions. A: Separation of mitochondrial proteins (5 μg) on 10% SDS-PAGE and staining with silver nitrate. B: Immunodetection of mitochondrial proteins (20 μg) with anti N-PDPc antibody, after electroblotting on nitrocellulose sheets. Lane 1: IM_1 fraction; lane 2: IM_2 fraction; lane 3: IM_3 fraction; lane 4: IM_4 fraction; lane 5: matrix space; lane 6: outer membranes.

3. Results

3.1. Purification of mitochondrial membranes

In an attempt to investigate the localization of the PDH phosphatase in mitochondria, we prepared sub-mitochondrial compartments, outer membranes, inner membranes and matrix space. Mouse liver mitochondria were purified using a mild ultra-sonic treatment, which eliminates contaminant membranes, as previously described by our group [10]. Total outer membranes were obtained after a controlled osmotic shock on purified mitochondria as described [10], followed by high speed centrifugation, while total inner membranes and matrix space were obtained after swelling of mitoplasts in 1 mM phosphate buffer and high speed centrifugation. Matrix space was constituted of soluble proteins released after this swelling and inner membrane vesicles, obtained in the pellet, were further purified on a discontinuous sucrose gradient. Four fractions of inner membrane vesicles were resolved after centrifugation and named fractions IM_1 to IM_4 from the top to the bottom of the gradient. According to the mitochondrial specific marker enzymes, monoamine oxidase, cytochrome *c* oxidase and citrate synthase, fractions IM_1 and IM_2 were characterized by the presence of these three enzymatic activities (Table 1), thus corresponding to vesicles where outer membrane and inner membrane were both present and closely associated forming probably transitional contact sites. The citrate synthase activity measured in fractions IM_1 and

Table 1
Marker enzyme activities in mitochondrial membrane fractions

Marker enzymes	Outer membranes	Inner membranes	Fraction IM_1	Fraction IM_2	Fraction IM_3	Fraction IM_4
Mitochondrial markers						
Monoamine oxidase	61.15	24.23	36.9	28.8	9.2	1.15
Cytochrome *c* oxidase	900	1880	2050	3800	2310	980
Citrate synthase	nd	250	170	230	340	240
Non-mitochondrial markers						
Basic 5′-nucleotidase	3.12	0.34	0.67	0.15	0.02	ND
Glucose 6-phosphate phosphatase	nd	11	115	7	ND	ND

Specific activities of monoamine oxidase, cytochrome *c* oxidase, citrate synthase, glucose 6-phosphate phosphatase are expressed as nmol min^{-1} mg^{-1}. Specific activity of basic 5′-nucleotidase is expressed as pmole min^{-1} mg^{-1}. ND: not detectable; nd: not determined.

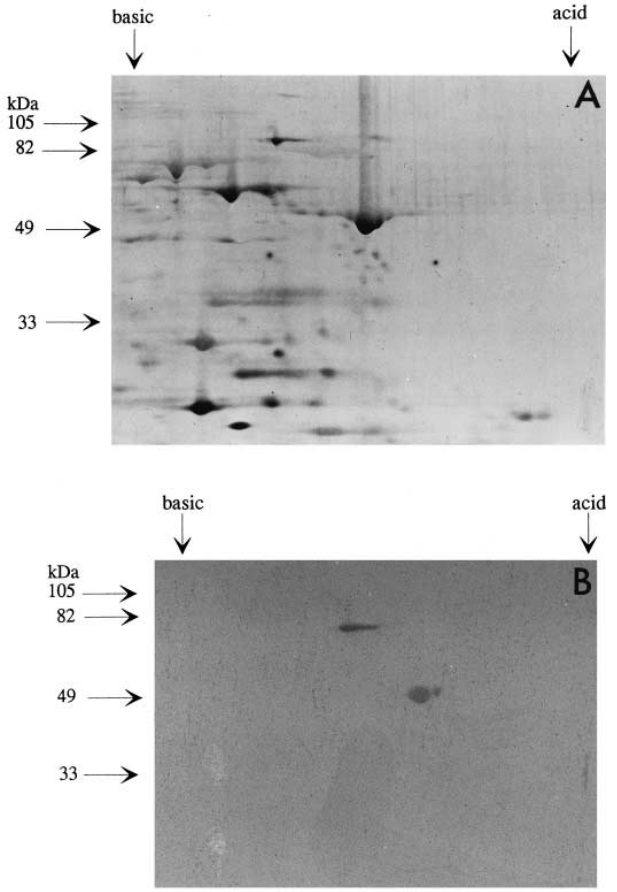

Fig. 2. 2D PAGE electrophoresis and Western blot analysis of sub-mitochondrial fractions. A: 2D-electrophoretic pattern of the IM_3 fraction: 30 μg of IM_3 fraction was analyzed by 2D SDS-PAGE (10% acrylamide) according to O'Farrel. Protein spots were visualized with silver nitrate. Pi were calibrated by the migration of standard proteins alone in the same conditions. B: 2D PAGE electrophoresis and immunoblotting of IM_3 fraction: 30 μg of IM_3 fraction were analyzed by 2D SDS-PAGE. After electrotransfer on nitrocellulose sheets, proteins spots were analyzed with the anti N-PDPc antibody.

IM_2 was probably due to the specific binding of this enzyme to the inner side of the inner mitochondrial membrane, in the low ionic strength conditions that were used for the swelling of mitoplasts [17], rather than reflecting a high content of matrix proteins in both fractions. Moreover, it is worth noting that enrichment of cytochrome *c* oxidase and citrate synthase activities increased in correlation with the decrease of monoamine oxidase activities in fractions IM_1 and IM_2 (Table 1). Fractions IM_3 and IM_4 contained almost exclusively citrate synthase and cytochrome *c* oxidase activities and were devoid of non-mitochondrial membranes and outer membranes, as specific activities of basic 5′-nucleotidase, glucose-6 phosphate phosphatase and monoamine oxidase were very low or undetectable (Table 1). These two fractions corresponded to vesicles of purified inner membranes containing matrix proteins, although we noted a decrease in cytochrome *c* oxidase activity. Fractions IM_3 and IM_4 were further used as pure inner membrane.

3.2. *Sub-mitochondrial localization of PDPc*

Each of the soluble or membranous sub-mitochondrial fractions was analyzed by SDS-PAGE using silver nitrate coloration (Fig. 1A) and by immunoblotting using a polyclonal antiserum raised against the amino-terminal sequence of the catalytic subunit of the PDH phosphatase, N-PDPc (Fig. 1B). We detected two specific proteins, one of 55 kDa which is the PDPc protein, and a protein of 80 kDa with hitherto unknown function (Fig. 1B). Both were present in all the inner membrane vesicles and were completely absent from the matrix space. However, on the one hand, the 80 kDa protein appeared to be less abundant in fraction IM_4 than in other lighter IM fractions and on the other hand, a protein of 58 kDa was detected at a low level in all IM fractions and in matrix space. This latter protein should be the imported form of the PDPc protein which possessed the mitochondrial import presequence [6]. Moreover, the outer membrane fraction contained only the 55 kDa protein, detected by the antibody. As this fraction corresponded to a total outer membrane fraction, without further separation on a sucrose gradient, it contained also inner membrane parts as previously described [10], which could account for the signal observed with the anti N-PDPc antibody. The antibody specificity was checked by pre-incubation of the antiserum with the immune peptide prior to the immunological clarification of proteins on the nitrocellulose sheet. The immunological detection was completely abolished by this treatment (data not shown). Moreover, control experiments performed either with pre-immune serum or with the second antibody alone did not permit any detection (data not shown). To further characterize the two specific mitochondrial proteins of 55 and 80 kDa, we analyzed them by 2D-electrophoresis and we chose fraction IM_3 which is a pure inner membrane fraction where both proteins were largely detected. Fraction IM_3 proteins were separated in a range of pH from 7 to 4.5 (Fig. 2A) and two spots were detected with the anti N-PDPc antibody; the 80 kDa protein had a Pi around pH 6, whereas the PDPc protein Pi was around pH 5 (Fig. 2B). This result is in agreement with the theoretical PDPc Pi of 5.5, calculated from its sequence. To gain further insight into the properties of these mitochondrial proteins, we studied their association with the inner membrane either by treatment of the membrane with increasing salt concentrations or by Triton X-114 temperature-induced phase separation. 25 μg of fraction IM_3 was incubated in the presence of several concentrations of NaCl. Membranous and solubilized pro-

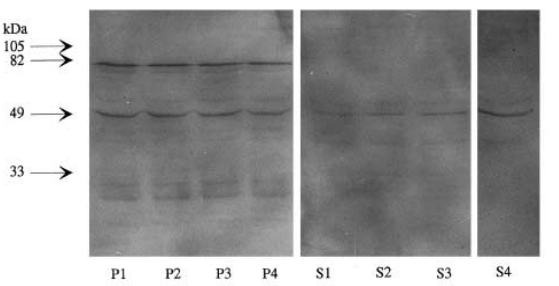

Fig. 3. Treatment of inner membrane with increased salt concentrations. Samples of IM_3 fraction (25 μg) were incubated for 30 min at +4°C in presence of increased concentrations of NaCl (from 50 mM to 500 mM). After centrifugation, soluble proteins (supernatant, lanes S) and membranous proteins (pellet, lanes P) were collected and analyzed by SDS-PAGE, immunoblotting and anti N-PDPc antibody revelation. P1/S1: 50 mM NaCl; P2/S2: 150 mM NaCl; P3/S3: 250 mM NaCl; P4/S4: 500 mM.

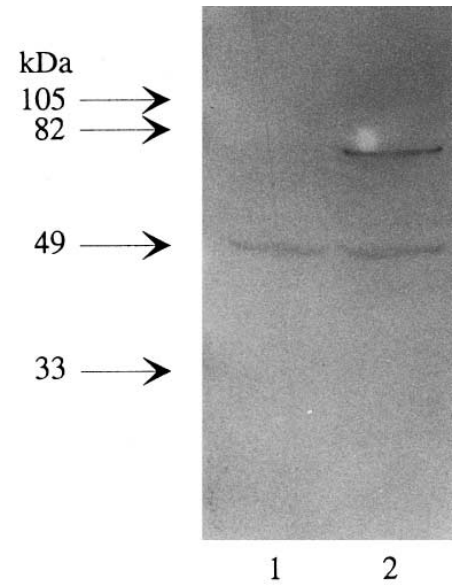

Fig. 4. Triton X-114 phase partitioning. A sample of IM_3 fraction (25 μg) was incubated for 10 min at 37°C and submitted twice to Triton X-114 phase partitioning. Soluble hydrophilic proteins (lane 1) and insoluble hydrophobic proteins (lane 2) were collected and analyzed by SDS-PAGE, immunoblotting and anti N-PDPc antibody revelation.

teins were analyzed by SDS-PAGE and immunoblotting with the anti N-PDPc antibody. The results (Fig. 3) showed that PDPc was slightly solubilized by a concentration of 50 mM NaCl (Fig. 3, lane S1) and that the solubilization increased with salt concentration (lanes S1–S4), correlating with a decrease of the detection of this protein in the membranous pellet (Fig. 3, lanes P1–P4). The efficiency of the salt solubilization of fraction IM was improved using NaCl concentrations up to 0.5 M. Even in these conditions, PDPc (protein of 55 kDa) did not appear completely as a soluble form, whereas the salt treatment did not succeed in solubilizing the 80 kDa protein (Fig. 3). Triton X-114 phase partitioning, which is a convenient method to distinguish between amphipathic, integral and hydrophilic proteins, was also achieved on inner mitochondrial membranes. After incubation at 37°C, hydrophilic proteins collected in the aqueous phase and hydrophobic proteins collected in the detergent phase were analyzed by SDS-PAGE and immunodetection was performed with the anti N-PDPc antibody. Fig. 4 showed that the PDPc protein was recovered after phase partitioning both in the aqueous phase (Fig. 4, lane 1) and in the detergent phase (Fig. 4, lane 2) with the same apparent detection intensity, whereas the 80 kDa protein was only detected in the detergent phase as a standard hydrophobic protein (Fig. 4, lane 2). No detection of this latter protein was observed in the aqueous phase (Fig. 4, lane 1).

4. Discussion

Up to now, very few studies concerning the purification of the PDP have been performed [2–5]. The procedure steps involved treatments such as freezing or sonication of the mitochondria, which are known to cause irreversible modification of protein/membrane organization, not allowing any conclusions about the PDP sub-localization in mitochondria. The aim of this study was therefore to purify and to characterize sub-mitochondrial compartments in order to specify the PDPc repartition in this organelle using a specific antibody. Our results clearly indicate that the catalytic subunit of the enzyme (protein of 55 kDa) was not present in the outer membrane or as a soluble protein in the matrix space but was widely distributed throughout the mitochondrial inner membrane. The same localization was also observed for a specifically detected 80 kDa protein of unknown function.

Moreover, it is worth noting that the PDPc protein presented a biphasic behavior during Triton X-114 phase partitioning, as the enzyme was recovered both in the aqueous phase and in the detergent-rich phase, in the same amount according to the intensity of the antibody detection. On the other hand, the same method showed that the 80 kDa protein was only present in the detergent-rich phase, as a hydrophobic protein [16]. In addition, the spot observed for the 55 kDa protein in 2D-electrophoresis led us to conclude that the 55 kDa proteins, recovered in both aqueous and detergent phases, were not two different proteins with the same apparent molecular weight and sharing sequence homology, but could be two different forms of the same protein. These two forms should differ by minor characteristics, which do not affect their electrophoretic mobility but are sufficient to modify their interaction with the inner mitochondrial membrane. Hydrolysis of a short peptide sequence, as previously described for another protein [18], or a post-translational modification such as acylation [19] could explain such results, which are also confirmed by the fact that only a part of the PDPc content was solubilized by incubation of inner membrane in 1 M NaCl (data not shown), the remainder remaining stably associated with the membrane.

The anti N-PDPc antibody used in this work led us first to prove the PDPc sub-localization in mitochondria and provides a powerful tool for further studies on PDP regulation, particularly in conditions of enzyme activation by a putative intracellular mediator in response to insulin action [20].

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